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Present and Future RNA-based Approaches to Medical Genomics

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Abstract

Nucleic acids-based technology is emerging as a valuable field that integrates research from science and technology to create novel nanodevices and nanostructures with various applicationsin modern nanotechnology. Nowadays, applicationsof RNA-based technologyare employed in biomedical and pharmaceutical research, biosensoring, nanopharmaceutics and others. It has been proven that RNA isa very suitable medium for self-assembly of diverse nanostructures, catalytic nanodevices and cell delivery systems.At the same time, genomics is becoming increasingly valuable for modern medicine due to the advancements made by second generation sequencing technologies. In this review, I discuss various applications of designer ribozymes and diverse RNA-based approaches to medical genomics. The areas discussed include RNA-based approaches for molecular sensoring and diagnostics, antibacterial drug discovery, exogenous control of gene expression, and gene silencing. These approaches havebecomepossible due to the advancement of various methods for engineering functional RNAsas well asthe discoveries made in RNA biology. Furthermore, different RNA-based antisense technologies are reviewed together with methods for nucleic acid delivery to the cell. The research that has been done so far in the field of RNA engineering hasa far-reaching impact on medical genomics, which isthe main focus of this review.

Keywords: RNA; Medical genomics; Allosteric ribozymes; Computational design; Molecular biosensors; Gene targeting; Drug development; Exogenous control of gene expression

Introduction

Nowadays, RNA-based research is emerging as a crossing point among the natural and engineering sciences. It is an area of exciting discoveries that includes many different scientific and technological fields. In fact, many naturalsciences, includingchemistry and biology along with many types of chemical and biological engineering at a molecular and sub-molecular level are involved in modern medicine. The progress achieved by the next generation sequencing (NGS) technologies [1] in recent years led to the discovery of novel targets for drug development and diagnostics. The interplay among RNA engineering, RNA biology, NGS technologies, and medical genomics creates new possibilities for drug development and molecular diagnostics. In this review, I presentcurrent and future RNA-based approaches to medical genomics as the focus set on drug development [2], molecular diagnostics [3] and forthcoming RNA-based therapeutic strategies [4].

As RNA-based biotechnology developed, its definition is leadingmore to the designof functionalsystemsthat can detect and react to molecular signals from the environment [5]. RNA technology is a rapidly improving field that involves several major discovers and technology developments. The discoveries include ribozymes and riboswitches [6], microRNAs and RNAi [7].The technology development in general, includes a creation of passive and active nanostructures, made of many interacting components or integrated nanodevices. The passive nanostructures are engineered to perform one particular task, whereas the active structures are designed to execute several different functions. Active nanostructures can be molecular sensors, actuators, drug delivery devices, and others. For instance, molecular sensors can detect the presence or the absence of specific molecules and pass predefined molecular signals to other sensors. Such molecular sensors can be used as report systems in many different applications, including drug discovery through high-throughput screening arrays. Moreover, molecular sensorscan be engineered to work as Boolean logic gates [8]. As a result, they can perform logical operations and solve computational problems [4]. Molecular logic gates can be designed to work together by passing signals among them in various circuits *in vitro* as well as *in vivo* [9].

In fact, RNA-based biotechnology is one of the fastest growing fields of research based on engineering nanosystems. It is an intersection between nanotechnology and biology. This paper discusses the main applications of designer nanostructures and nanodevices based on RNA molecules. In fact, one of the first nanostructures was made of RNA using the expertise accumulated in recombinant technology and molecular biology over the last three decades. RNAhave been proven to be suitable nanoscale materials. They are relatively easy to synthesize, amplify, detect, and modify. They can be used both *in vitro* and *in vivo*. Therefore, RNA engineering plays a very important role in modern nanobiotechnology.

The main advantages of making structures and devices with RNA are the possibilities of applying many established engineering methods in conjunction with various tools of molecular biology and nucleic acid chemistry. In fact, it is easy to chemically synthesize DNA oligonucleotides and to obtain synthetic RNAs by *in vitro* transcription using double-stranded (ds) DNA templates. Moreover, RNAare easy to amplify, detect, store, and modified. There are powerful software programs that enable researchers to design automatically specific RNAmolecules with desired properties.

There are three distinct methods for designing RNA-based biosensors. They include *in vitro* selection [10], rational design [11], and computational methods [8]. RNA sensors have many different applications in nanobiotechnology. Such applications include

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molecular computing [12,13], reporter systems for high-throughput screening assays for antibacterial drug discovery [5,14] synthetic gene control elements for exogenous regulation [4]. We discuss these applications in the next sections of this paper.

Engineering Allosteric Ribozymes as Reporter Systems in HTS Arrays for Drug Discovery

The initial methods applied for engineering functional nucleic acids were based on various *in vitro* selection procedures. As a result, the first RNA aptamers were obtained. The word "aptamer" comes from the Latin "aptus" - fit, and Greek "meros" - part. They have the ability to bind various ligands with high specificity by forming complex three-dimensional structures.The first nucleic acids based aptamers were obtained by an *in vitro* selection method called systematic evolution of ligands by exponential enrichment (SELEX) [15]. In fact, the aptamers are still produced by various *in vitro* selection procedures.

These findings suggested that nucleic acids, and particularly RNA, could be very capable molecular sensors. This makes some researchers to speculate that RNA molecules may directly sense the presence of different metabolites in the cell without involvement of any proteins. In fact, the bid to find such natural RNA aptamersled to the discovery up to now of 17 different classes of RNA sensors termed riboswitches and this number is still growing [16]. These newly discovered gene control elements are usually found in the 5' untranslated region (UTR) of messenger (m) RNAs (Figure 1a). They enable mRNAs to adjust their expression in the presence of specific metabolites [17].

Genome-wide searches revealed that riboswitches are present mainly in bacteria. In addition, a few riboswitch classes are also found to control gene expression in some eukaryote species by alternative splicing. The genome-wide search of bacterial riboswitches has important applications to medical genomics. This research results in the discovery of many riboswitch classes in over 40 different human pathogenic bacteria [2]. This opens brand new avenues for targeting new RNAs in antibacterial drug development [18]. The world-wide demand for new antibiotics is increasing due to the emerging of many resistant to the common antibiotics stains of human pathogenic bacteria. Targeting bacterial riboswitches may result in the discovery of novel classes of antibiotics that have few resistant strains or any for the time being because there are not yet developed antibiotics specific targeting bacterial riboswitches.

The riboswitches consist of a ligand-binding (natural aptamer) domain and an expressionplatform. There are various riboswitch-dependent expression mechanisms, including transcription termination, prevention of translation, mRNA destabilization, and others [2]. Riboswitches are classified in different classes according to their aptamer domains, which have conserved secondary and tertiary structures. Note that one type of riboswitch can have different expression platforms. For instance, the FMN riboswitch regulates gene expression either by transcription termination or by prevention of translation [19]. In both cases, however, the aptamer domain of the FMN riboswitch has the some conserved structure that forms highly specific pocket for FMN. As a result of FMN binding, the flavin riboswitch alters its structure, which leads to transcription termination or prevention of translation.

The development of high-throughput screening [20] assays based on bacterial riboswitches is an essential part in this process. It is important to create HTS compatible RNA sensors in which the ligand-binding part of a riboswitch is fused to a reporter domain. Such HTS compatible reporter systems can be obtained by fusing the aptamer domain of a riboswitch (Figure 1a) with a ribozyme. Ribozymes are conserved RNA molecules, which possess a catalytic function [21]. For our aim, we can employ the minimal version of the hammerhead ribozyme (Figure 1b and c). It is called in this way because its specific secondary structure formed by three stems that looks like a hammerhead [22]. It cleaves itself at the position indicated by the arrowhead (Figure 1b and c). The minimal version of the hammerhead ribozyme (Figure 1b) is a short RNA molecule that requires a high concentration of Mg^{2+}(10 mM) to exhibit its catalytic function. In contrast, the extended version of the hammerhead ribozyme (Figure 1c) works at physiologically-relevant concentration of Mg^{2+} (1 mM) due to its canonical and non-canonical interactions between the loop in stem II and the bulge loop in stem I [23]. Therefore, the minimal version of the hammerhead ribozyme is used *in vitro* while the extended form is employed *in vivo*.

The minimal version of the hammerhead ribozyme can be fused in stem II with a riboswitch aptamer to obtain different allosteric sensors (Figure 1d). For this goal, we have used computational methods based on modeling secondary structures or tertiary interactions in the presence and in the absence of the ligand. These approachesare very accurateand time efficient allowing many different riboswitch classes to be adjusted for HTS assays based on fluorescence detection.We employ a fluorescence resonance energy transfer (FRET) method for detection of ribozyme cleavage [14] under multiple turnover conditions. In the FRET method, two fluorescent dyes are employed. One is attached to the 5' end while the other is on the 3' end. One dye serves as a reporter (Figure 1e, R) that emits a fluorescence signal upon excitation while the other one is a quencher (Figure 1e, Q), which absorbs the emitted signal. However, if the FRET-labeled RNA molecule is cleaved the R and Q are not anymore in close proximity and the quencher cannot absorb the emitted by the reporter fluorescence signal.

To make use of such detection system, we have engineered allosteric hammerhead ribozyme that cleave external FRET-labeled RNA molecules by opening stems I and III of the ribozyme (Figure 1d and e). When an effector molecule is not present stem II is not formed and, therefore, the ribozyme is inactive (Figure 1d). In contrary, when a molecule specifically binds to the aptamer domain stem II of the ribozyme is formed (Figure 1e). This activates the ribozyme, which cleaves its substrate RNA (Figure 1e, in red). This ribozyme serves as a Boolean logic gate with YES function.As a result, the quencher and reporter are unconnected and the fluorescence emitted by the reporter can be detected (Figure 1f). This approach can be fully automated in a HTS format testing hundreds of thousands chemical compound for specific binding to the aptamer domain of any riboswitch. This can be used as a first step in riboswitch-based antibacterial drug discovery. The application of microfluidic technology may further advance this process [2,24].

RNA-based Circuits

The hammerhead ribozyme can be used to create allosteric molecular sensors with various Boolean logic function, including NOT, YES, OR, and AND [8]. In addition, the ribozyme can be designed to sense not only small molecules but also specific oligonucleotide DNA and RNA molecules. Moreover, the ribozymes can be programmed to passsignals from one to other within a molecular circuit. Such molecular circuits can be used for various applications, including molecular computing and diagnostics *in vitro*, and for building synthetic signaling pathways *in vivo*. For instance, such a circuit was demonstrated by two oligonucleotide-sensing ribozymes termed YES-1 and YES-2 (Figure

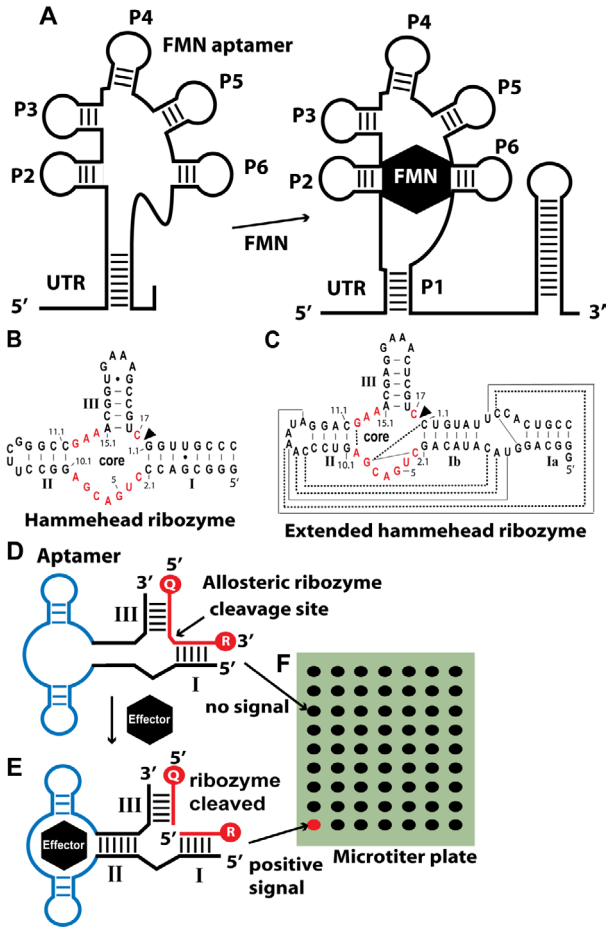

Figure 1: Engineering allosteric ribozymes as reporter systems in HTS arrays for drug discovery. (A) Supposed secondary structure of the aptamer domain of the flavin riboswitch in the absence and presence of FMN. (B) The sequence and the secondary structure of the minimal version of the hammerhead ribozyme. The arrowhead indicates the cleavage site. (C) The sequence and the secondary structure of the minimal version of the extended hammerhead ribozyme from the human parasite Schistosomamansoni, which exhibit high-speed of cleavage due to its canonical and non-canonical interactions between the nucleotides of stem II and these of the bulge loop of stem I. (D) Allosteric ribozymes can be employed as biosensors which recognize small molecules and cleave external RNA by fusing an aptamer domain (in blue) with a minimal version of the hammerhead ribozyme. As detection system can be used a florescence resonance energy transfer (FRET) method. In this method the external RNA, cleaved by the ribozyme, is attached to a quencher (Q) in its 5' end and to a reporter (R) in its 3' end. If the effector is not present, the allosteric ribozyme is inactive, and the RNA substrate will not be cleaved. (E) If the effector molecule is present, it binds to the allosteric domain, so that stem II is formed and the ribozyme will be activated. The activated ribozyme cleaves the external RNA, which leads to separation of the R from the Q. (F) In this case, we will detect positive florescence signal. High-throughput machines can be used to perform this array on microtiter plates.

2). Both ribozymes were designed by a computational procedure [8] that is a subject for a pending patent application. The computational procedure for designing oligonucleotide-sensing allosteric ribozymes is superior to *in vitro* selection methods both in terms of time efficiency and design accuracy.

In the absence of effector oligonucleotides both YES ribozymes are designed to be in inactive state since stem IV is formed instead of stem II (Figure 2a). In the presence of oligonucleotide effector-1, the YES-1 ribozyme is activated since stem II is formed instead of stem IV when a specific oligonucleotide effector binds the oligonucleotide binding site (OBS) of the ribozyme (Figure 2b). As a result, the YES-1 ribozyme cleaves itself and released a RNA oligonucleotide that serves as an effector for the YES-2 ribozyme. This results in the activation of YES-2 ribozyme, which cleaves itself (Figure 2c).

Note that the effector-1 that triggers the circuit can be a DNA as well as RNA molecule. The ribozymes are very specific. They can distinguish perfectly well two mismatches over the length of 22 nt. In addition, several oligonucleotide-sensing allosteric ribozymes can be designed to work sin parallel. This makes them suitable molecular sensors for various molecular diagnostic applications.

Integratedrna Based Nanodevices with a Complex Logic Function as a Tool for Molecular Diagnostics

Integrated nanodevices made of nucleic acidscan combine

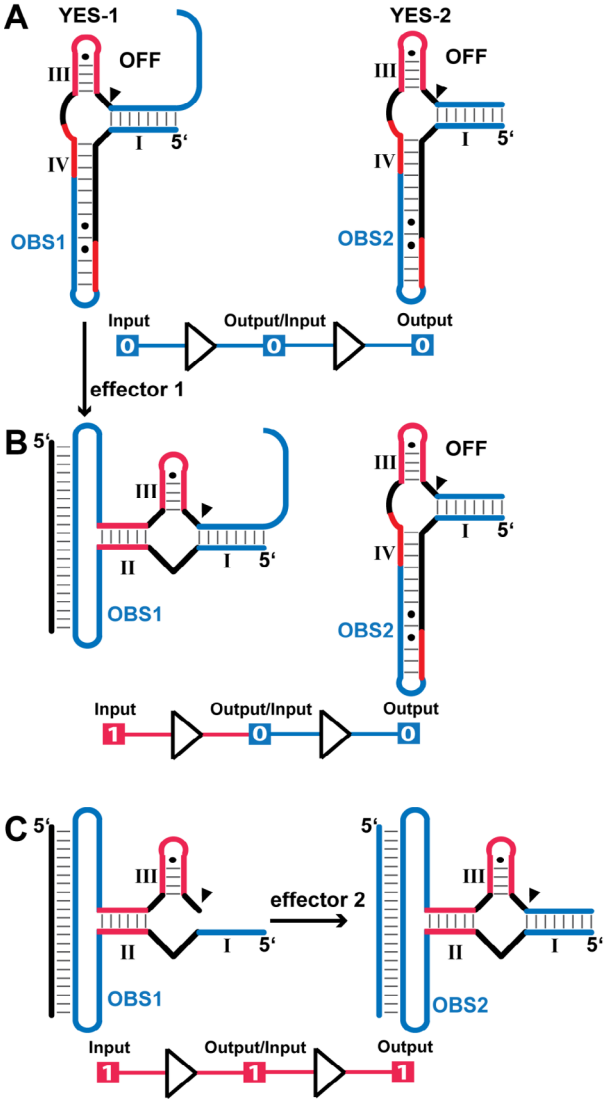

Figure 2: Molecular computing circuits. (A) The secondary structures of two allosteric ribozymes with YES logic function are depicted. In the absence of an effector molecule, both ribozymes are inactive. (B) When effector 1 is present, it binds to the oligonucleotide binding site (OBS1) of the first ribozyme that activates it. As a result, the YES-1 ribozyme undergoes self-cleavage. (C) The 3' cleavage fragment (effector 2), released after the self-cleavage of YES-1, targets the second ribozyme. As a result, YES-2 is also activated.

the function of several molecular logic gates in a single functional molecule or in an assembly of molecules. Therefore, such devices can work as integrated circuits at a nanoscale level. There are some conceptual, designing, and engineering obstacles that need to be overcomebeforeroutineproduction ofintegrateddevices with nucleic acids, including RNA.For the time being, there are a few nucleic acids based devices that integrate the functionality of several logic gates and can be used for diagnostic purposes. However, even now, there are examples that provide experimental validation on the feasibility of engineering such nanodevices.

An example for a RNA-based device that can sense the length of its target RNA molecules was recently described [3]. This nanodevice has the complexity of an integrated circuit and can serve as three input AND Boolean logic gate (Figure 3). Such gate has a true output signal only in the presence of all three inputs. Our ribozyme-based three-input logic gate is capable of sensing the length of its target RNA molecules. It has two OBSs that can sense two domains of a target RNA molecule. They are embedded into stem II of the minimal version of the hammerhead ribozyme. This ribozyme is designed to cleave an external RNA molecule that has two effector domains and a substrate domain that are complementary to the OBSs of stem II and the substrate binding site (SBS) of the ribozyme that forms stem I and III. In fact, if the spacer between the effector domains and the SBS of the ribozyme is too short the forms stems I and III will not be formed and the target RNA will not be cleaved. In contrast, if the spacer of the target RNA molecule is long enough stems I and III will be properly formed. As a result the target RNA will be cleaved.

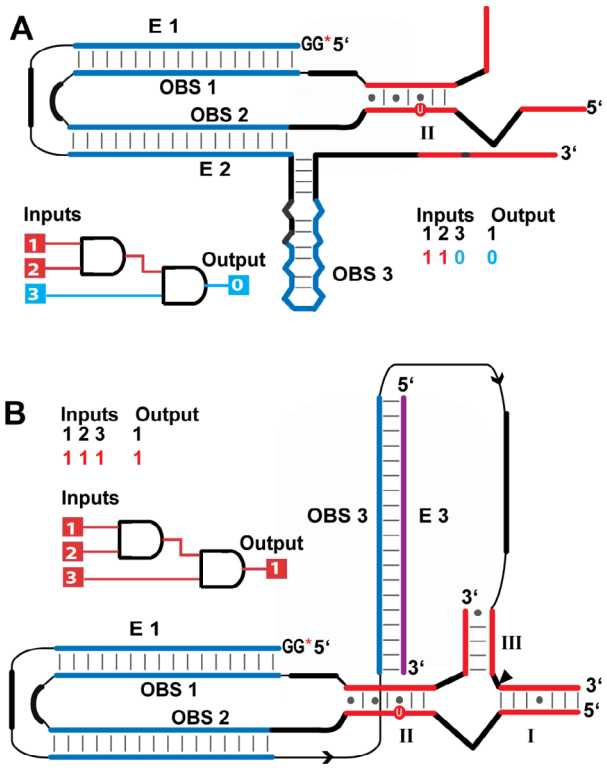

Figure 3: Integrated RNA based nanodevice that sense the length of target RNAs. (A) The secondary structure of a three input logic gate with AND logic function is depicted. If the target RNA molecule is too short the stems I and III are not formed and the ribozyme is inactive. (B) When the target molecule is long enough all three stems of the ribozyme are formed the substrate RNA is cleaved.

There are many genetic diseases that are associated with triplet repeat expansion in mRNAs [25] such as the Huntington's disorder. In all these disorders, the mutant mRNA molecules are longer than the normal ones. The triplet repeats tend to form stable hairpin structures that makes the length detection of such triplet repeat expansions very difficult (Figure 3a). To overcome this problem, we can open the hairpin structure by heating it in the presence of antisense oligonucleotide. As a result, the hairpin will form a double-strained structure. In this case if the target mRNA is long enough stems I and III will be formed (Figure 3b). Therefore, the target RNA will be cleaved.This approach can be used for diagnostic purposes by seeking and destroying specific RNA molecules with a certain length that are indicative for certain triple repeat expansion disease.

Allosteric Ribozymes as Designer Cis-Acting Gene Control Elements

There are many disorders that are associated with abnormal expression of certain RNA molecules in the cell. We could oligonucleotide-sensing ribozymes that can detect such abnormal expression of important RNAs in the cell and to react by altering expression of exogenous proteins that can overcome the disorder. In this way, we can achieve two main functions for prevention and curing various diseases. In the first place, we can monitor the emerging of the disease indicative RNAs in real time. In the second place, we can take immediate actions upon detection of such RNAs. This is a future therapeutic strategy based on allosteric ribozymes that work as cis gene control elements in the cell.

We can embed high-speed oligonucleotide-sensing ribozymes at the 3' end of synthetic mRNAs expression in the cell by viral vectors (Figure 4). If the designer ribozyme is a NOT gate, it will cleave itself in the absence of disease indicative RNA in the cell. As a result, the synthetic mRNA will be decayed and will be not translated into a protein (Figure 4a). In contrast, in the presence of disease indicative RNA the ribozyme will be deactivated. The synthetic mRNA will be translated and therefore, the desired protein will be expression, which will prevent the disease's development. For instance, if there are cancer related RNAs in the cell we could induce the expression of exogenous p53 that could kill the cancer cell [4].

We may also sense health indicative RNAs in the cell and expressed a certain protein only in the absence of such RNAs. To achieve this we need to employ an allosteric ribozyme with YES logic function as depicted in (Figure 4b). Note that all high-speed allosteric ribozymes are based on the extended version of the hammerhead ribozyme (Figure 1c). In this case, the OBS is introduced in stem III to preserve the interactions between stems I and II that are responsible the *in vivo* function of the ribozyme.

Gene Silencing Techniques via Trans-Acting Ribozymes

In modern pharmaceutical research, all tools, which are manufactured at a nanoscalelevelare termed nanopharmaceutics. There are two basic types of nanopharmaceutics. One type of them is those where therapeutic molecules themselves act as drugs. The other type includes nanocarriers that are used fordrugdelivering. In this case, the drugs are directly coupled to the nanocarriers. There are various methods for drug attachment to nanocarriers such as functionalizing, loading, and encapsulating. As nanocarriers can be used polymerparticles, nanobeads, nanotubes, and quantumdots. Nanodrugs [26] can be produced in various forms such as nanosuspension, nanoemulsion, nanogels, and nanospheres.

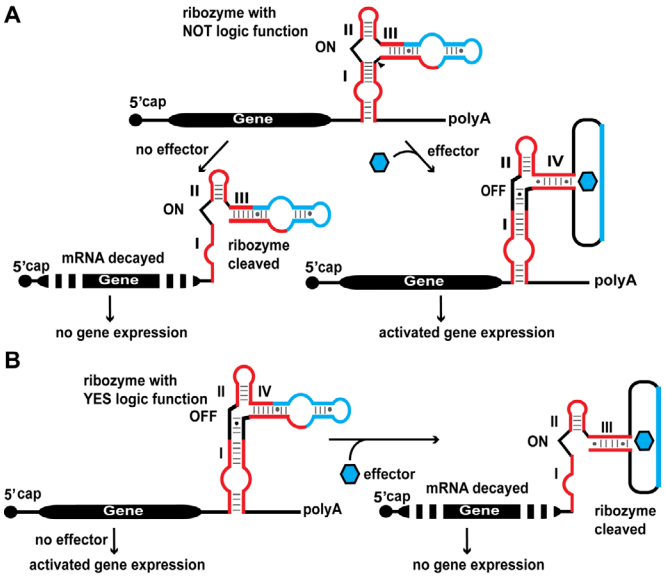

Figure 4: Ribozyme-mediated regulation of gene expression in eukaryotes. The gene expression in eukaryotes can be regulated through destabilization of mRNAs controlled by allosteric ribozymes. We can insert a designer ribozyme in the 3' UTR of mRNA that encodes a desired protein. If the ribozyme is activated, it undergoes self-cleavage that leads to mRNA decay. (A) An allosteric ribozyme with NOT logical function can be inserted in the 3' UTR. In the absence of effector, it cleaves itself, which destabilizes the mRNA. When a specific ligand is present, the ribozyme is inactivated. The mRNA remains stable and is translated into protein. (B) When a ribozyme with YES logical function is inserted in the 3'-UTR, it will be inactive in the absence of effector, and the gene expression of our protein will be activated. If the effector is present, it will activate the ribozyme. As a result, the mRNA will be decayed and there will be not protein synthesis.

It is believed that the nanopharmaceutical research may be very beneficial for improving drug stability and delivery. Nanopharmaceutics offer some important advantages such as improved bioavailability, specific molecular targeting, and controlled release. In addition, nanopharmaceutics can be engineered to pass intracellular compartments and the blood to brain barrier andto protect fragile drugs.They have many different ways of administration including ocular, pulmonary, oral, topical, parenteral,nasal, and intravenous.

In some cases, the usage of nanopharmaceutics results in reducing toxicity. However, some nanocarriers have been found to be toxic in certain ways of administration. This has to be well studied to avoid adverse effects when applying novel nanopharmaceutics. Nanocarriers are particulate systems with various sizes between1 and 1000 nm.They have been successfully used in the treatment of various diseases. Manytypes of nanocarriers have been introduced up to now. They include polymermicelle, dendrimers, liposomes, and quantum dots.

RNA molecules can be delivered in the cell by various types of liposomes. In addition, they can be expressed in the cell by different types of viral vectors. In fact, nucleic acids show immense potential to treat cancer, acquired immune deficiency syndrome, neurological diseases and other incurable human diseases. Intracellular delivery of nucleic acids is facilitated by nanovectors, both viral and non-viral.A major advantage of non-viral vectors over viral vectors is safety. Therefore, RNA molecules can be delivered in many different ways in the cell.

The advancements of these methods will further empower the application of various ribozyme-based gene silencing techniques as novel therapeutics.We can employ high-speed allosteric ribozymes with YES logic function (Figure 5a) for conditional expression of short hairpin (sh) RNAs only in the presence of some specific RNAs such as viral RNAs (Figure 5b and c). The shRNAs expressed in the cell will be cut by the dicer (Figure 5d) and will enter a RNA interference (RNAi) pathway [27,28]. They can work as microRNAs preventing the translation of specific mRNAs related to certain disease development or can decay some target mRNAs in conjunction to the RISC complex (Figure 5e). Note that these processes tolerate some mismatches and, therefore, can affect several different mRNAs simultaneity. In fact, one shRNA sequence usually targets many mRNAs in the cell and therefore it silences many different genes. That's why, in most cases, we cannot afford to constantly expressed shRNAs. Instead we need to express shRNAs only under certain conditions and for limited time. Such future approach could deal with this problem. In addition, we can design a high-speed ribozyme to cleave some specific RNA in the cell (Figure 5f). However, this approach will be not as efficient as shRNAs because it will have limited turnovers. All these ribozyme-based gene silencing techniques may have potent applications to medical genomics by targeting specific genes *in vivo*. The future development of RNA delivery systems in the cell will play a key role in successful applications of these methods.

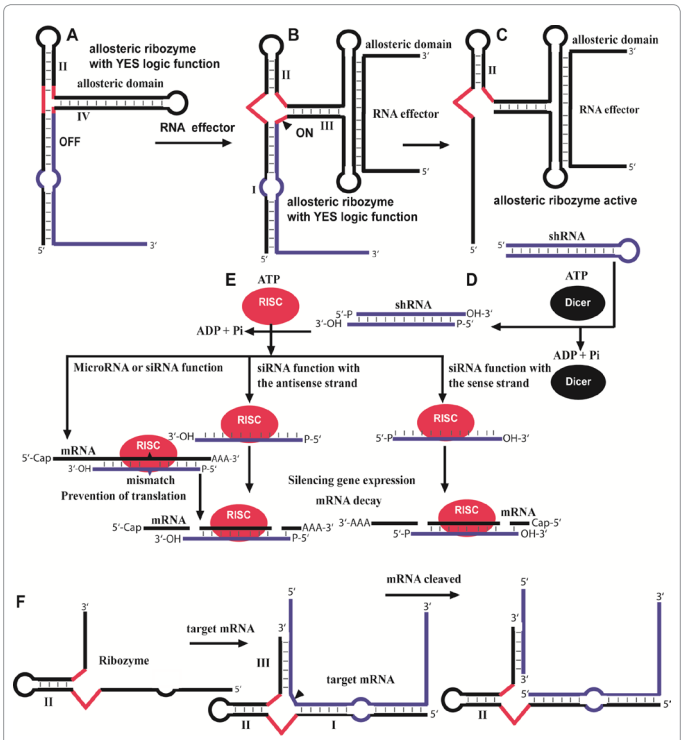

Figure 5: Ribozyme-based gene silencing techniques. (A) Without an effector RNA the YES gate is designed to fold into an inactive structure where a stem IV is formed instead of stem III. (B) The YES switch binds the effector RNA and folds into an active state in which stem III is formed. (C) As a result the ribozyme undergoes self-cleavage and shRNA is produced. (D) Dicer converts shRNA into dsRNA with sticky ends. (E) In the presence of RISC complex the dsRNA is single-stranded and used for gene repression either by microRNA function via translational suppression prevention of RNA translation by RNA decay. When there is a mismatch(s) with the target mRNA the microRNA function is often executed. However, mRNA decay is observed not only with perfectly matching dsRNA but also in the presence of some mismatches. (F) High-speed ribozyme can be designed to directly cleave a specific RNA in the cell.

Conclusions

RNA basedresearchhas proven to be a productive field of modern science and technologythat has many important applications to discovery of novel molecular targets for drug development and diagnostics, creating reporter systems in HTS arrays, allosteric biosensors, integrated nanodevices, designer gene control elements, drug delivery, and others. Moreover, the discovery of new mechanisms for control of gene expression such as microRNAs, RNAi, and riboswitches gives us not only better understanding for the complexity of the RNA function within the cell but also provides us with bigger opportunities for applying novel RNA-based engineering strategies both *in vitro* and *in vivo*. The advancement of RNA biology and engineering, on one hand, and the progress achieved in the medical genomics by the HTS machines, on another hand, offer new exciting opportunities for developing of novel therapeutic strategies for tackling various forms of cancer, viral infection diseases, deployment of novel antibiotics, and others. In addition, RNA engineering promises to create original gene therapies by reprogramming the cell fate. All this would be possible if the medical genomics discovers RNAs that are important targets and/or markers in a disease development process. Our abilities to identity such RNAs by NGS technologies will improve over time due to advancement of sequencing technology.

Future Prospective

The application of novel safe and efficient viral expression vectors will be critical for reaching clinical trials of these methods in the future. The development of more effective nanoparticle methods for cell delivery of siRNAs will increase our abilities to develop various RNAi based therapeutic approaches. There will be some years to come before the RNA-based technology matured and become a widely spread in medicine. The proper interaction between the RNA engineering and medical genomics can significantly speed up this process. The progress achieved in the computational design of allosteric ribozymes may play a pivotal role in developing various strategies for disease-dependent reprograming of cell fate in the near future together with our ability to specifically inhibit the expression of targeting mRNAs in the cell. In addition, targeting bacterial riboswitches may lead to the development of novel classes of antibiotics with less resistance pathogenic bacterial strains.

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